(12) UK Patent Application (19) GB (11) 2 324 529 (13) A

(43) Date f A Publication 28.10.1998

(21) Application No 9803559.5

(22) Date of Filing 20.02.1998

(30) Priority Data
(31) 038656 (32) 21.02.1997 (33) US

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(51) INT CL[6]
C07K 1/04 5/10 , C12Q 1/37

(52) UK CL (Edition P)
C3H HA3 H304 H380
G1B BAB B121 B734
U1S S1333

(56) Documents Cited
EP 0533226 A WO 96/33268 A WO 94/00154 A
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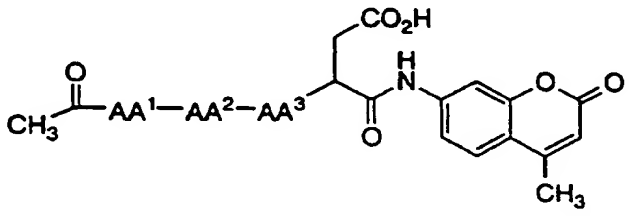
(58) Field of Search
ONLINE: CAS ONLINE

(54) Abstract Title

A combinatorial library based on a tetrapeptide substituted with aminomethylcoumarin for characterizing proteases

(57) A tetrapeptide substituted at the C-terminus with 7-amino-4methylcoumarin (MCA) and acetylated at the N-terminus is used to generate a positional scanning synthetic combinatorial library for the investigation of the substrate specificity of cysteine and serine proteases. Preferably the compound has a C-terminal aspartic acid having the formula I:



wherein AA1-AA3 are amino acids or substituted amino acids. The library preferably comprises mixture of at least 200 compounds of formula I. The analysis of Interleukin-1β converting enzyme (ICE, caspase I) substrate specificity is described.

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TITLE OF THE INVENTION
COMBINATORIAL LIBRARY AND METHOD FOR CHARACTERIZING PROTEASES

BACKGROUND OF THE INVENTION

Apoptosis is a form of cell death that is essential for morphogenesis, tissue homeostasis, and host defense (for review see R. E. Ellis, et al., Annu. Rev. Cell Biol., 7, 663-698 (1991)). There is accumulating evidence that defects in apoptosis may lead to several pathologies including some neurodegenerative disorders, ischemic injury, and cancer, such as Alzheimer's, Parkinson's and Huntington's diseases, immune deficiency and autoimmune disorders, ischemic cardiovascular and neurological injury, alopecia, leukemias, lymphomas and other cancers, which therefore makes the control of apoptosis an important potential target for therapeutic intervention (Kerr, et al., Br. J. Cancer, 26, 239-257 (1972); Martin, et al., Trends Biochem. Sci., 19, 26-30 (1994); Barr, et al., Biotechnology, 12, 487-493 (1994); Carson, et al. Lancet, 341, 1251-1254 (1994); C. B. Thompson, Science, 267, 1456-1462 (1995)). The discovery that CED-3, the product of a gene necessary for programmed cell death in the nematode *Caenorhabditis elegans* (*C. elegans*), is related to the cysteine protease interleukin-1β converting enzyme (ICE, caspase-1), established proteases to be key mediators in this process (J. Yuan, et al., Cell, 75, 641-652 (1993)). Interleukin-1β (IL-1β) is a major mediator of chronic and acute inflammation. Interleukin-1β converting enzyme (ICE/caspase-1, EC 3.4.22.36) is the cysteine protease responsible for the production of IL-1β in monocytes. Since its discovery in 1989, this enzyme has been the subject of intense interest as a potential therapeutic target for the treatment of inflammatory diseases. Although the precise biochemical pathways involved in mammalian cell death remain ill-defined, it is now clear that various proteases play an essential role in both the initial signaling events, and in the downstream processes that result in the apoptotic phenotype. Those that are known to be involved include members of the ICE/CED-3 or caspase (E. S. Alnemri, et al., Cell, 87, 171 (1996)) family of cysteine proteases, and the cytotoxic

lymphocyte-deriv d serine proteas , granzyme B. To date, ten caspases of human origin have been identified (ICE, ICH-1, CPP32, ICE_{rel}-II, ICE_{rel}-III, Mch2, Mch3, FLICE, ICE-LAP6, Mch4). A unified nomenclature for these enzymes has recently been adopted, using the term "caspase" as a root for serial names (E. S. Alnemri, et al., Cell, 87, 171 (1996)). A phylogenetic analysis of the caspases suggests that these enzymes may be grouped into two, possibly three, subfamilies.

Positional scanning synthetic combinatorial libraries (PS-SCL) are a powerful method for rapidly identifying high-affinity peptide sequences from a mixture of compounds (Pinilla, et al., Biotechniques, 13, 901-905 (1992); U.S. Patent No. 5,556,762). Peptide-based positional scanning synthetic combinatorial libraries are generally composed of several sub-libraries in which one position is defined with an amino acid, while the remaining positions contain a mixture of amino acids present in approximately equimolar concentrations (Ostresh, et al., Biopolymers, 34, 1681-1689 (1994)). Analysis of the library will identify the preferred amino acids for each position. Such libraries have been successfully employed for the identification of potent receptor ligands (Dooley, et al., Life Sciences, 52, 1509-1517 (1993); Wallace, et al., Peptide Research, 7, 27-31 (1994)), enzyme inhibitors (Owens, et al. . Biochemical and Biophysical Research Communications, 181, 402-408 (1991); Eichler, et al., Biochemistry, 32, 11035-11041(1993); Pinilla, et al., Drug Development Research, 33, 133-145 (1994); Eichler, et al. Peptide Research, 7, 300-307 (1994)), and specific antigens (Pinilla, et al., Biotechniques, 13, 901-905 (1992)), Pinilla, et al., Biochem. J., 301, 847-853 (1994)), but they have not previously been used to determine the specificity of a protease enzyme.

In an embodiment of the present invention, a positional scanning synthetic combinatorial library was employed to study the roles of these proteases in apoptosis, to identify appropriate fluorogenic substrates, and to facilitate inhibitor design, peptide substrate specificities. In accordance with the present invention, a positional scanning substrate library may be employed to identifying protease specificity and determine the amino acid preferences for serine or cysteine protease enzymes, such as human caspases, including ICE,

CED-3 and granzyme B. Using the present method, the complete specificity of a protease can be readily mapped in the length of time required to execute one assay. Through the use of the present invention, it was determined that the optimal tetrapeptide recognition sequence for ICE is WEHD, not YVAD, as previously believed.

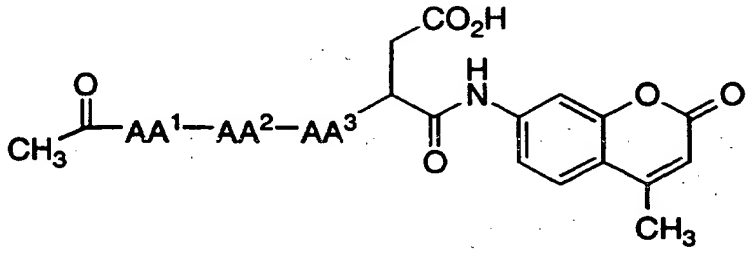
SUMMARY OF THE INVENTION

The present invention is directed to the use of positional scanning synthetic combinatorial libraries as research tools to identify the substrate specificity of a cysteine or a serine protease enzyme. In particular, the combinatorial libraries and the compounds of the present invention may be used to identify the substrate specificity of a cysteine or a serine protease enzyme.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to the use of positional scanning synthetic combinatorial libraries to identify the substrate specificity of a cysteine or a serine protease enzyme.

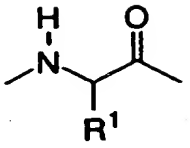
In one embodiment, the present invention is directed to compounds of the formula:



I

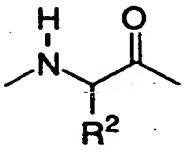
wherein:

AA^1 is an amino acid of formula AI:



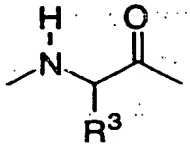
AI

AA^2 is an amino acid of formula AII:



AII

AA^3 is an amino acid of formula AIII:



AIII

wherein R^1, R^2 and R^3 are each independently selected from the group consisting of:

(a) hydrogen,

(b) C_{1-6}alkyl or substituted C_{1-6}alkyl, wherein the substituent is selected from:

(1) hydroxy,

(2) halo,

(3) -S-C_{1-4}alkyl,

(4) -SH

(5) C_{1-6}alkylcarbonyl,

(6) $-CO_2H$,

(7) $-CONH_2$,

(8) amino carbonyl amino,

(9) C_{1-4}alkylamino, wherein the alkyl is optionally substituted with hydroxy, and the amino is substituted with hydrogen or carbobenzyloxy,

(10) guanidino,

(11) C_{1-4}alkyloxy,

(12) phenyl C_{1-4}alkyloxy,

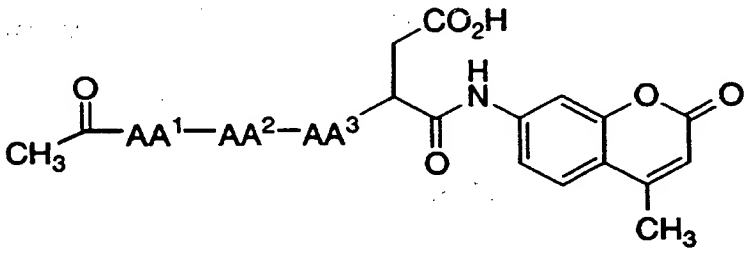
(13) phenyl C_{1-4}alkylthio,

(14) C_{1-6}alkyloxycarbonyl, and

(c) aryl-C_{1-6} alkyl, wherein aryl is phenyl, 1- or 2-naphthyl, 9-anthracyl, or 2-, 3- or 4- pyridyl, and wherein the aryl may be mono and di-substituted, wherein the substituent is independently selected from: C_{1-6}alkyl, halo, hydroxy, C_{1-6}alkyl amino, C_{1-6}alkoxy, C_{1-6}alkylthio, and C_{1-6}alkylcarbonyl;

and pharmaceutically acceptable salts thereof.

Preferred compounds of the present invention include those of formula I:



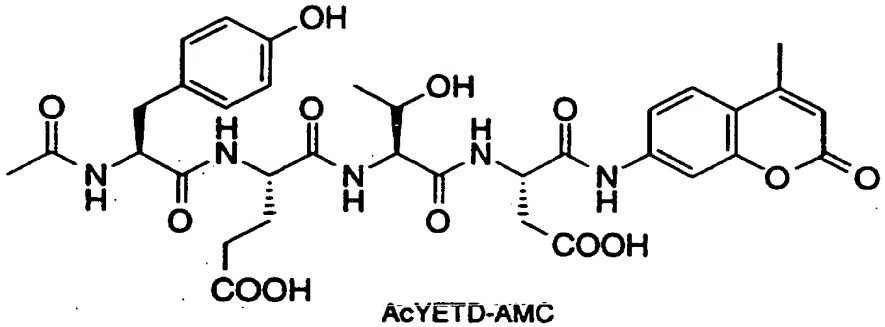
I

wherein:

AA^1, AA^2 and AA^3 are each independently selected from the group consisting of: the L- and D- forms of the amino acids including glycine, alanine, valine, leucine, isoleucine, norleucine, serine, threonine, aspartic acid, asparagine, glutamic acid, glutamine, lysine, hydroxy lysine, histidine, proline, hydroxy proline, arginine, phenylalanine, tyrosine, tryptophan, cysteine, methionine, β-alanine,

ornithine, homoserine, homotyrosine, homophenylalanine, aminoisobutyric acid and citrulline.

Specific compounds of the present invention include:



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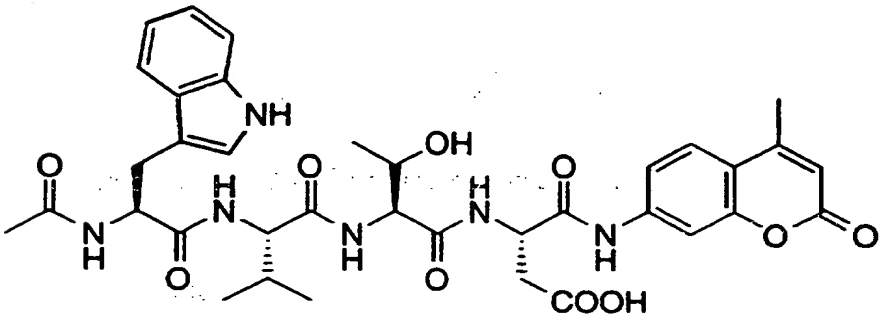

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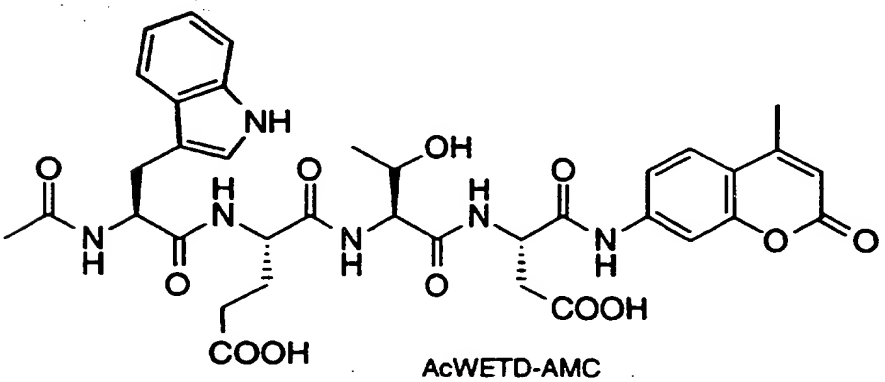

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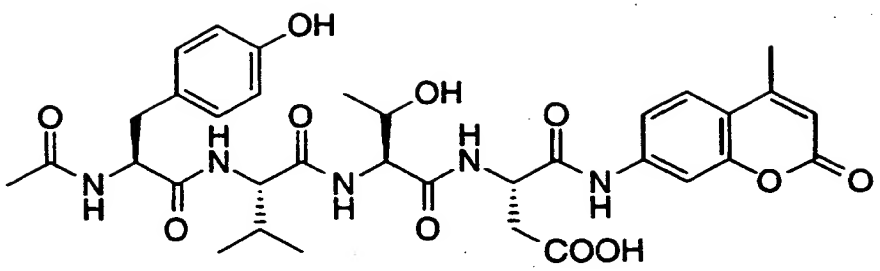

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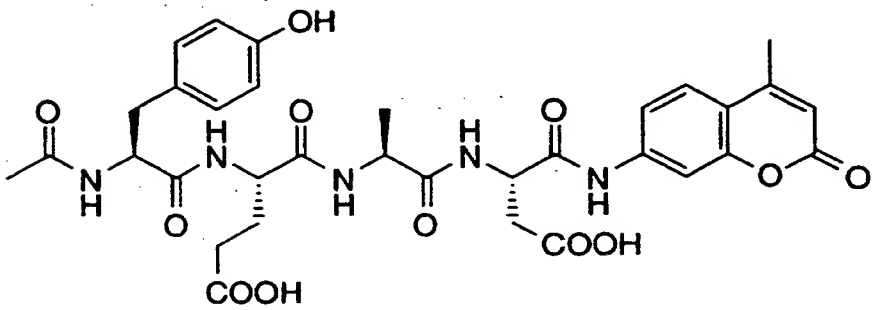

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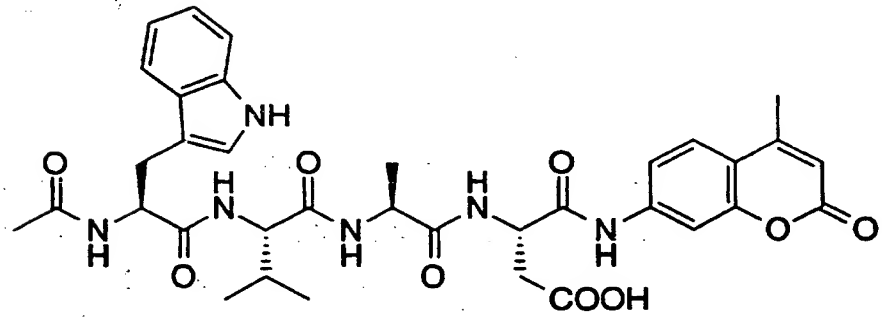

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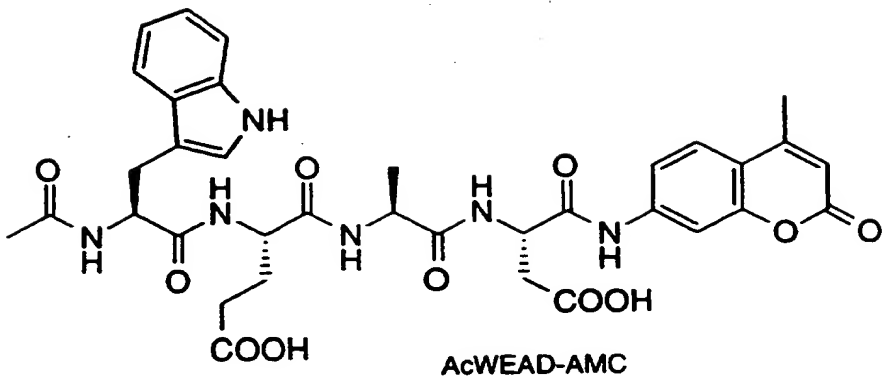

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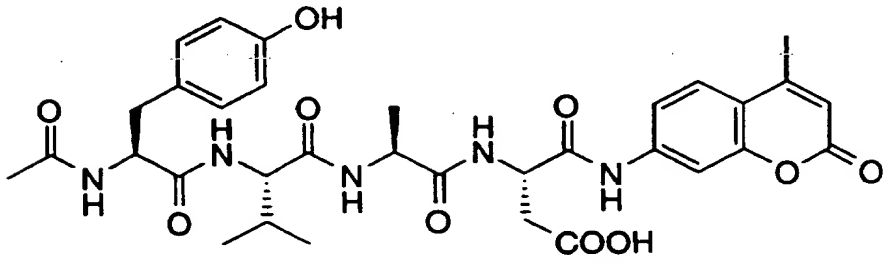

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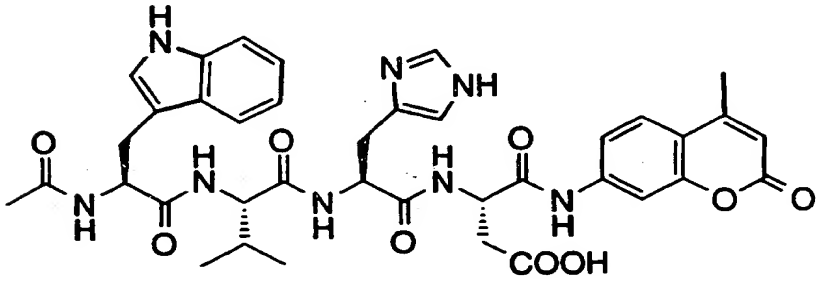

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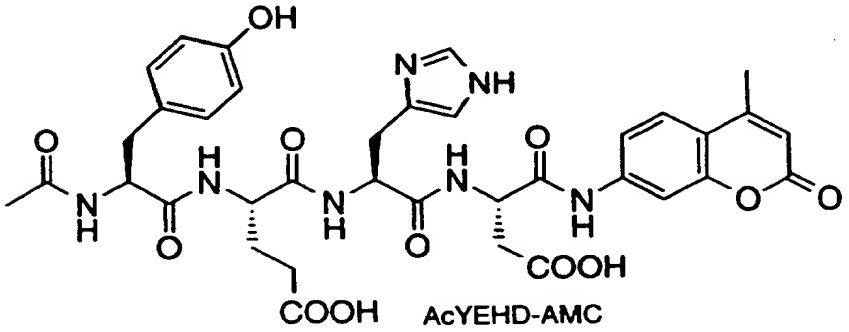

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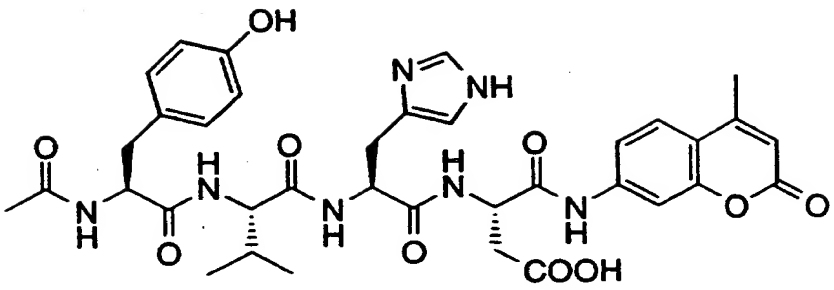

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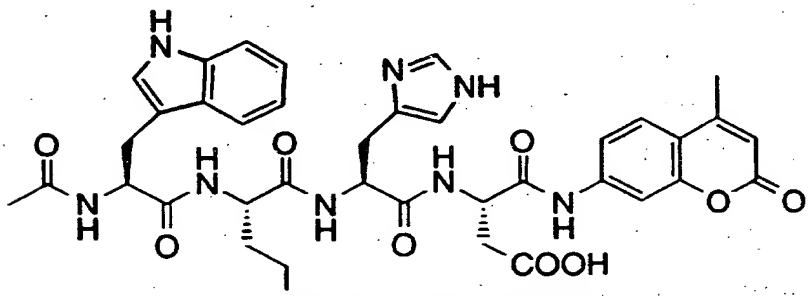

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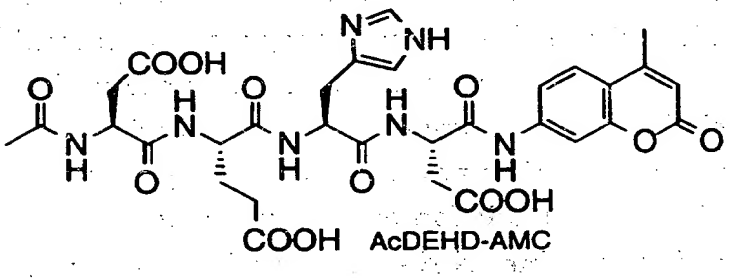
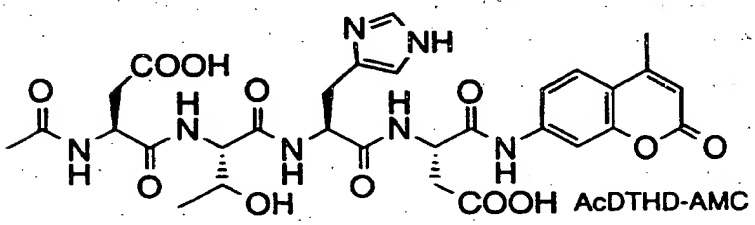


AcDEHD-AMC



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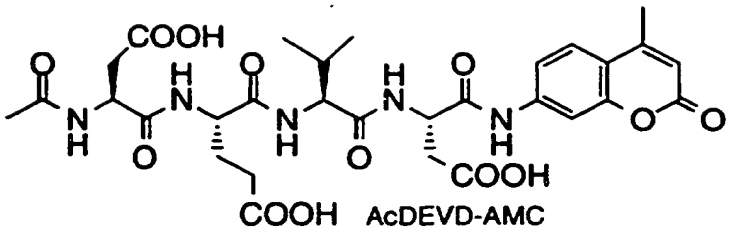


and pharmaceutically acceptable salts thereof.

The combinatorial libraries and compositions of the present invention comprise a compound of formula I as an active ingredient or a pharmaceutically acceptable salt, thereof, and may also contain a pharmaceutically acceptable carrier and optionally other therapeutic ingredients. The term "pharmaceutically acceptable salts" refers to salts prepared from pharmaceutically acceptable non-toxic bases including inorganic bases and organic bases. Salts derived from inorganic bases include aluminum, ammonium, calcium, copper, ferric, ferrous, lithium, magnesium, manganic salts, manganous, ammonium, potassium, sodium, zinc and the like. Particularly preferred are the calcium, magnesium, potassium, and sodium salts. Salts derived from pharmaceutically acceptable organic non-toxic bases include salts of primary, secondary, and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines, and basic ion exchange resins, such as arginine, betaine, caffeine, choline, N,N'-dibenzylethylenediamine, diethylamine, 2-diethylaminoethanol, 2-dimethylaminoethanol, ethanolamine, ethylenediamine, N-ethyl-morpholine, N-ethylpiperidine, glucamine, glucosamine, histidine, hydrabamine, isopropylamine, lysine, methylglucamine, morpholine, piperazine, piperidine, polyamine resins, procaine, purines, theobromine, triethylamine, trimethylamine, tripropylamine, tromethamine, and the like.

When the compound of the present invention is basic, salts may be prepared from pharmaceutically acceptable non-toxic acids, including inorganic and organic acids. Such acids include acetic, benzenesulfonic, benzoic, camphorsulfonic, citric, ethanesulfonic, fumaric, gluconic, glutamic, hydrobromic, hydrochloric, isethionic,

lactic, maleic, malic, mandelic, methanesulfonic, mucic, nitric, pamoic, pantothenic, phosphoric, succinic, sulfuric, tartaric, p-toluenesulfonic acid, and the like. Particularly preferred are citric, hydrobromic, hydrochloric, maleic, phosphoric, sulfuric, and tartaric acids.

In another embodiment, the present invention is directed to a combinatorial library which comprises a mixture of compounds of the formula I. It is preferred that the combinatorial library comprise a mixture of at least 50 compounds of the formula I. It is more preferred that the combinatorial library comprise a mixture of at least 100 compounds of formula I. It is even more preferred that the combinatorial library comprise a mixture of at least 200 compounds of formula I.

The term "combinatorial library" refers to a set of compounds having an oligomeric modular structure, wherein all members of the library are formed by essentially identical reactions (differing only in the reactants added), where the diversity of the library results from the combination of a variety of monomers in several positions within the molecule. Combinatorial libraries of the present invention may achieve a much greater degree of diversity due to the ready availability of compounds which can be easily coupled in positions within formula I. A combinatorial library may include mixtures of compounds, or may consist entirely of individual compounds. The term "mixture" refers to an aliquot of compounds, either in solution or bound to a particular solid phase, which may be assayed simultaneously.

In another embodiment, the present invention embraces the use of a positional scanning synthetic combinatorial library to identify the substrate specificity of a cysteine or a serine protease enzyme. A positional scanning synthetic combinatorial library can be used to determine the precise substrate preferences of a protease enzyme, to predict and test high-affinity inhibitors of a protease enzyme, and to measure the enzymatic activity of the protease enzyme in basic research studies, diagnostic assays and drug screening assays.

A method for identifying the substrate specificity of a cysteine or a serine protease enzyme comprises the steps of:

(a) providing a number of sets of soluble, unsupported mixed oligopeptides bearing a spectrophotometric or fluorogenic leaving group, wherein the number of sets is equal to the number of amino acid positions within each oligopeptide,

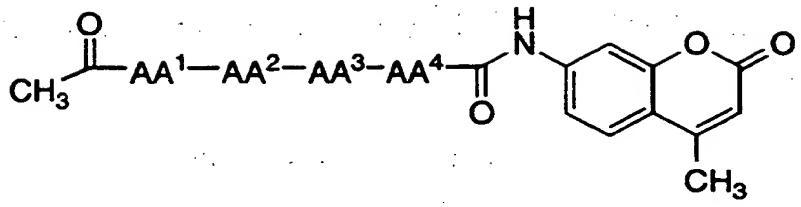
wherein each set of oligopeptides is comprised of subsets of oligopeptides, wherein each subset of oligopeptides is comprised of oligopeptides in which a predetermined position of the oligopeptide chain contains a fixed amino acid and in which the remaining positions of the oligopeptide chain contain a mixture of amino acids present in approximately equimolar concentrations;

(b) separately mixing each subset from one of the sets with a cysteine or a serine protease enzyme in an aqueous media, separately determining the production of the spectrophotometric or fluorogenic leaving group, and ascertaining the one or more subsets of the set that provide for the production of the spectrophotometric or fluorogenic leaving group; and

(c) repeating step (b) using the subsets from each of the remaining sets, wherein the identity and position of the amino acid residue of each one or more subsets of the set that provide for the production of the spectrophotometric or fluorogenic leaving group gives the substrate specificity of the cysteine or the serine protease enzyme.

In a preferred embodiment, the present invention is directed to the use of a combinatorial library comprised of a mixture of compounds of the formula I to identify the substrate specificity of a cysteine or a serine protease enzyme.

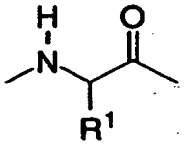
In an alternate embodiment, the present invention is directed to the use of a combinatorial library to identify the substrate specificity of a cysteine or a serine protease enzyme, wherein the combinatorial library is comprised of a mixture of compounds of the formula II:



II

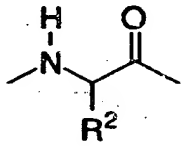
wherein:

AA^1 is an amino acid of formula AI:



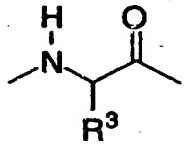
AI

AA^2 is an amino acid of formula AII:



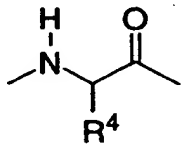
AII

AA^3 is an amino acid of formula AIII:



AIII

AA^4 is an amino acid of formula AIV:



AIV

wherein R^1, R^2, R^3 and R^4 are each independently selected from the group consisting of:

(a) hydrogen,

(b) C_{1-6}alkyl or substituted C_{1-6}alkyl, wherein the substituent is selected from:

(1) hydroxy,

(2) halo,

(3) -S-C_{1-4}alkyl,

(4) -SH

(5) C_{1-6}alkylcarbonyl,

(6) -CO_2H,

(7) -$CONH_2$,

(8) amino carbonyl amino,

(9) C_{1-4}alkylamino, wherein the alkyl is optionally substituted with hydroxy, and the amino is substituted with hydrogen or carbobenzyloxy,

(10) guanidino,

(11) C_{1-4}alkyloxy,

(12) phenyl C_{1-4}alkyloxy,

(13) phenyl C_{1-4}alkylthio,

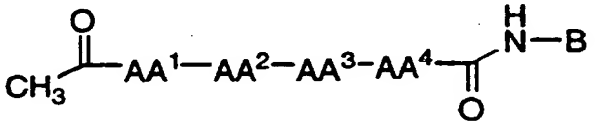
(14) C_{1-6}alkyloxycarbonyl, and

(c) aryl-C_{1-6} alkyl, wherein aryl is phenyl, 1- or 2-naphthyl, 9-anthracyl, or 2-, 3- or 4- pyridyl, and wherein the aryl may be mono and di-substituted, wherein the substituent is independently selected from: C_{1-6}alkyl, halo, hydroxy, C_{1-6}alkyl amino, C_{1-6}alkoxy, C_{1-6}alkylthio, and C_{1-6}alkylcarbonyl;

and pharmaceutically acceptable salts thereof.

In the compounds of formula II it is preferred that AA^4 is aspartic acid.

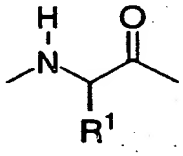
In an alternate embodiment, the present invention is directed to the use of a combinatorial library to identify the substrate specificity of a cysteine or a serine protease enzyme, wherein the combinatorial library is comprised of a mixture of compounds of the formula III:



III

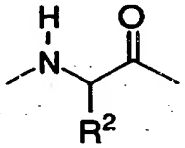
wherein:

AA^1 is an amino acid of formula AI:



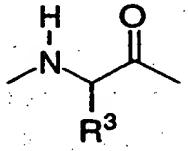
AI

AA^2 is an amino acid of formula AII:



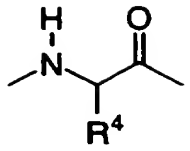
AII

AA^3 is an amino acid of formula AIII:



AIII

AA^4 is an amino acid of formula AIV:



AIV

wherein R^1, R^2, R^3 and R^4 are each independently selected from the group consisting of:

(a) hydrogen,

(b) C_{1-6}alkyl or substituted C_{1-6}alkyl, wherein the substituent is selected from:

(1) hydroxy,

(2) halo,

(3) -S-C_{1-4}alkyl,

(4) -SH

(5) C_{1-6}alkylcarbonyl,

(6) -CO_2H,

(7) -$CONH_2$,

(8) amino carbonyl amino,

(9) C_{1-4}alkylamino, wherein the alkyl is optionally substituted with hydroxy, and the amino is substituted with hydrogen or carbobenzyloxy,

(10) guanidino,

(11) C_{1-4}alkyloxy,

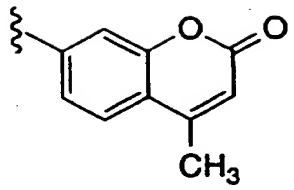
(12) phenyl C_{1-4}alkyloxy,

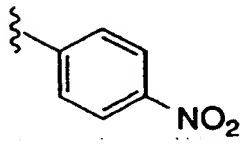
(13) phenyl C_{1-4}alkylthio,

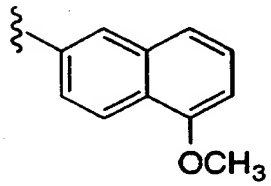
(14) C_{1-6}alkyloxycarbonyl, and

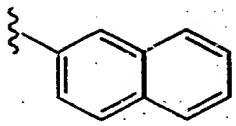
(c) aryl-C_{1-6} alkyl, wherein aryl is phenyl, 1- or 2-naphthyl, 9-anthracyl, or 2-, 3- or 4- pyridyl, and wherein the aryl may be mono and di-substituted, wherein the substituent is independently selected from: C_{1-6}alkyl, halo, hydroxy, C_{1-6}alkyl amino, C_{1-6}alkoxy, C_{1-6}alkylthio, and C_{1-6}alkylcarbonyl;

B is a spectrophotometric or fluorogenic leaving moiety selected from the group consisting of:







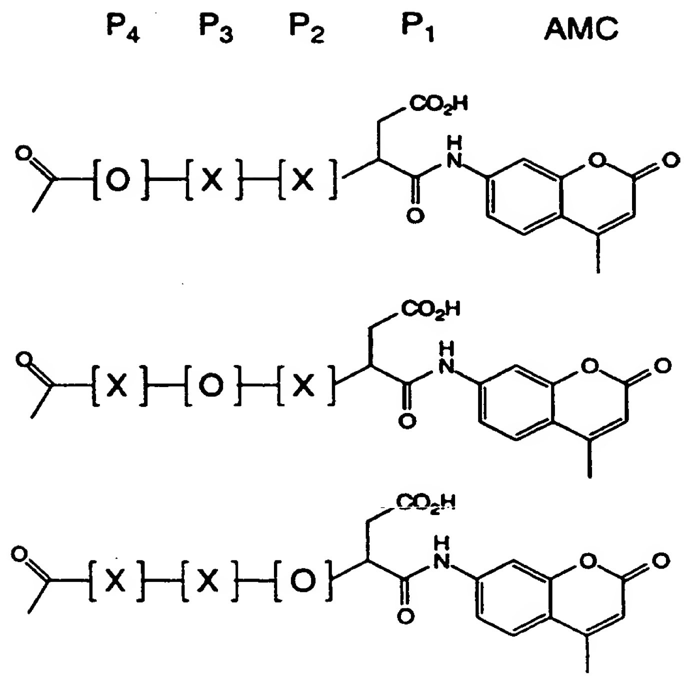


and pharmaceutically acceptable salts thereof.

In the compounds of formula III it is preferred that AA^4 is aspartic acid.

The combinatorial libraries of the present invention may be used as research tools to identify the substrate specificity of a cysteine or a serine protease enzyme. The substrate may be peptidal or non-peptidal in nature.

For example, to better understand the biological function(s) of Interleukin-1β converting enzyme (ICE), and to facilitate development of selective inhibitors, its substrate specificity was determined in detail using a positional scanning synthetic combinatorial libraries with the general structure Ac-X-X-X-Asp-AMC
(AMC = aminomethylcoumarin).



This particular positional scanning synthetic combinatorial library is composed of 3 separate sub-libraries of 8,000 compounds each. In each sub-library, one position ("O") is defined with an amino acid and represents the spatially addressed amino acids, while the remaining two positions ("X") represent a mixture of amino acids (excluding cysteine) present in approximately equimolar concentrations. Using this strategy, analysis of the three sub-libraries (20 samples each) affords a complete understanding of the amino acid preferences across S_2, S_3, and S_4 subsites. Aspartic acid was held constant at P_1 because of the near absolute specificity ICE family members exhibit for this residue. The Asp-AMC (aminomethylcoumarin) moiety was linked to the solid support by the beta-carboxyl of the Asp residue at P_1 via a Mitsunobu reaction (Hughes, D.L., Org. Reactions, 42, 335 (1992)). The remaining tripeptide was constructed employing standard Fmoc amino acid chemistry.

In order to confirm that the present invention provides a reliable measure of specificity, it was used it to determine the amino acid preferences of caspase-1/ICE. The results of these studies indicated that the preferred recognition motif for ICE is WEHD, and led to the

synth sis of a fluorogenic substrate, Ac-WEHD-AMC, that is cleaved 50-fold more efficiently than that previously believed to be optimal for this enzyme, with a second-order rate constant (k_{cat}/K_m) of 3.3×10^6 $M^{-1}s^{-1}$. The corresponding tetrapeptide aldehyde, Ac-WEHD-CHO, was found to have a K_i for inhibition of caspase-1 of 56 pM, making it the most potent reversible, small-molecule inhibitor described for any caspases. Similarly, the present invetion was used to determine the substrate specificity of other protease such as apopain or human caspases, including ICE, CED-3 and granzyme B. These results clearly demonstrated that the method of the present invention furnishes an accurate identification of primary specificity that can be exploited to produce efficient substrates and potent inhibitors of cysteine proteases and serine proteases.

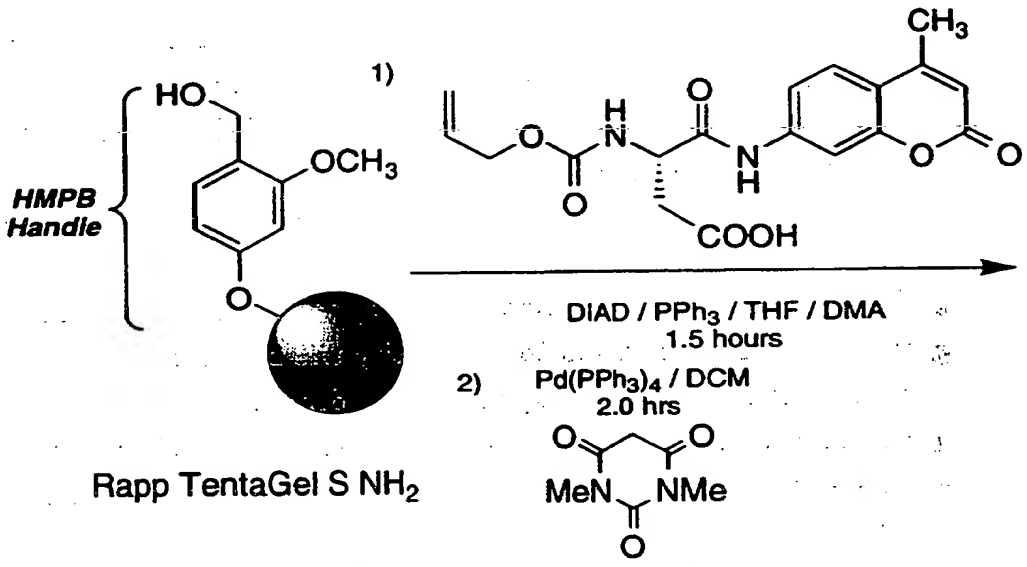
As will be clear to one skilled in the art, the positional scanning synthetic combinatorial library disclosed herein has utility in determining the specificities of caspases other than ICE. The instant invention may be used to to identify the substrate specificity of a cysteine or a serine protease enzyme. Accordingly, the present invention is directed to the use of these compounds and methods in the preparation and execution of screening assays for compounds which are substrates for a cysteine or a serine protease enzymeprotease enzymes. For example, the design of the specific library for ICE is based on several catalytic features that appear to be conserved among all known ICE homologues, including a near absolute requirement for Asp in the S_1 subsite and an ability to accommodate large hydrophobic groups (e.g. aminomethylcoumarin) in the prime sites.

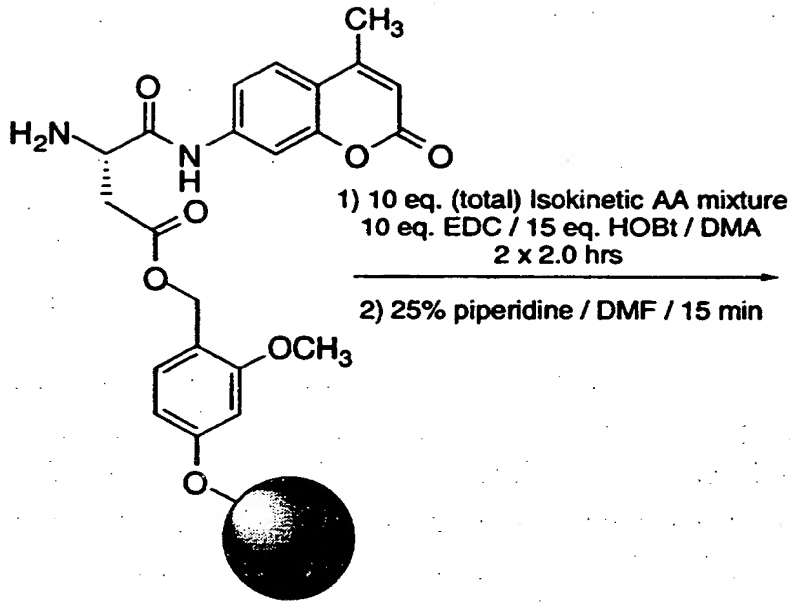
The present invention is the first example of a positional scanning synthetic combinatorial library that has been used to determine protease specificity. Presumably, this approach has not been more broadly applied for this purpose because of the difficulty involved in measuring hydrolysis rates of peptides in mixtures. Using the strategy employed here, in which a fluorescent leaving group is incorporated in the P_1' position, reaction rates are measured simply by following an increase in fluorescence. The results obtained suggest that an analogous library in which the P_1 position is also varied (Ac-X-X-X-X-

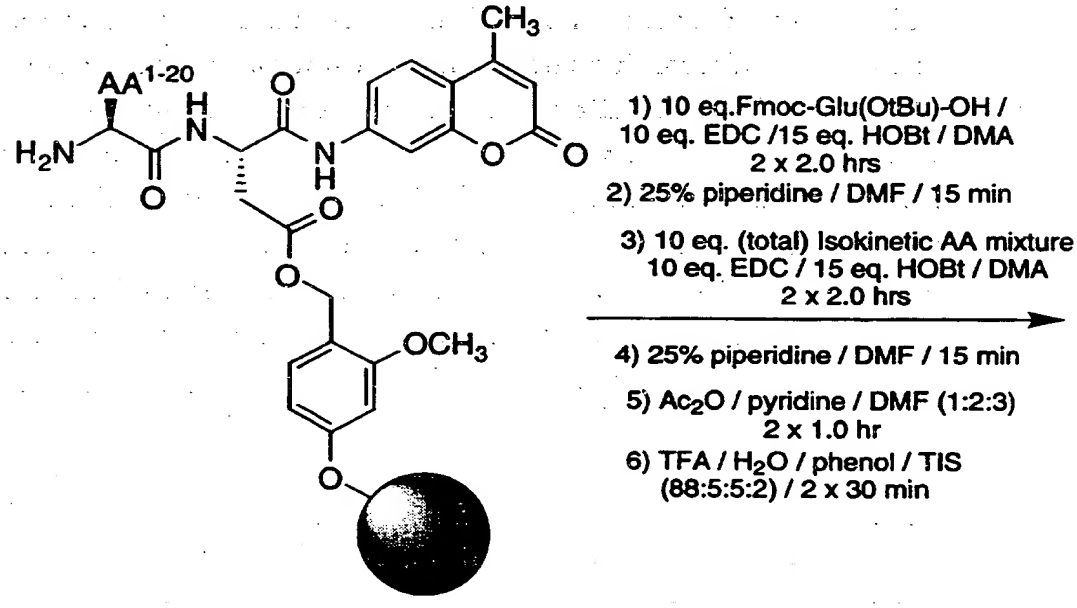
AMC) would be invaluable for determining the specificity of many cysteine and serine proteases; in general these enzymes have th ability to cleave peptide analogs containing a photometric leaving group in P1'.

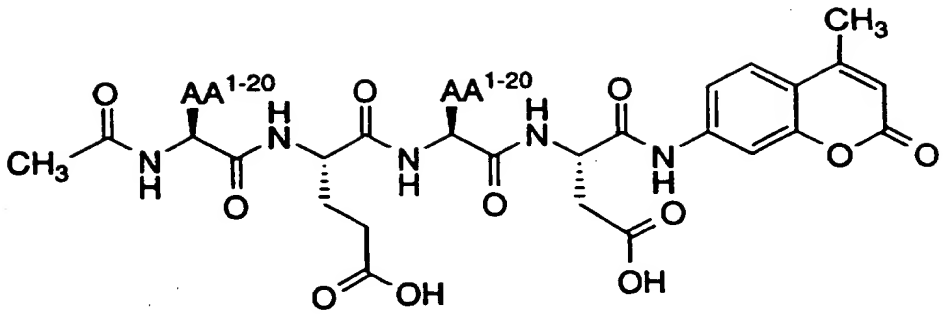
The compounds of the instant invention are prepared using the procedures depicted generally in the following Schemes and more explicitly described in the Examples.

SCHEME 1




CH3
H2N
O
N
H
O
O
O
O
OCH3
O
1) 10 eq. (total) Isokinetic AA mixture
10 eq. EDC / 15 eq. HOBt / DMA
2 x 2.0 hrs
2) 25% piperidine / DMF / 15 min

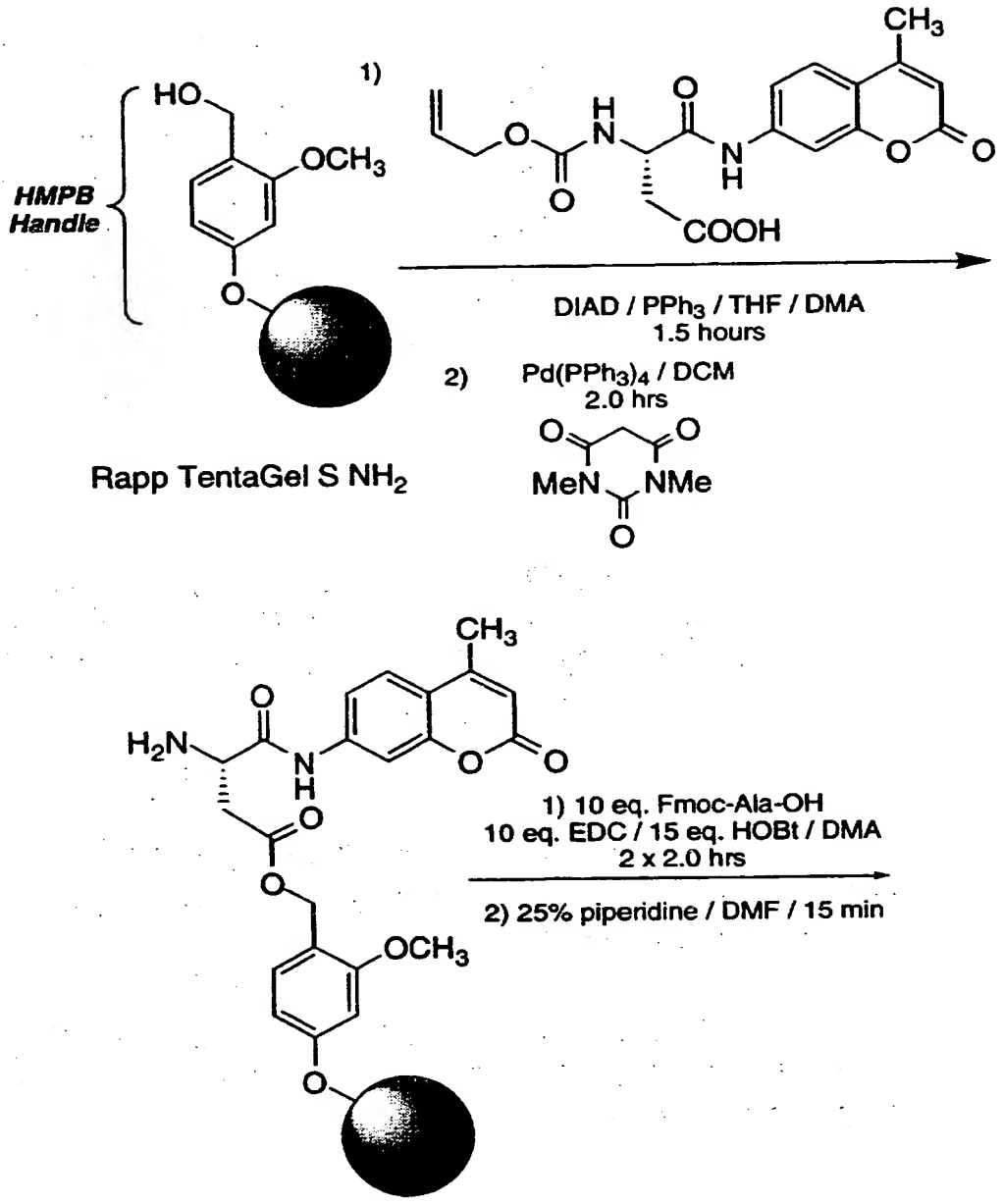

CH3
AA1-20
H2N
H
N
O
O
N
H
O
O
O
O
OCH3
O
1) 10 eq.Fmoc-Glu(OtBu)-OH /
10 eq. EDC /15 eq. HOBt / DMA
2 x 2.0 hrs
2) 25% piperidine / DMF / 15 min
3) 10 eq. (total) Isokinetic AA mixture
10 eq. EDC / 15 eq. HOBt / DMA
2 x 2.0 hrs
4) 25% piperidine / DMF / 15 min
5) Ac2O / pyridine / DMF (1:2:3)
2 x 1.0 hr
6) TFA / H2O / phenol / TIS
(88:5:5:2) / 2 x 30 min

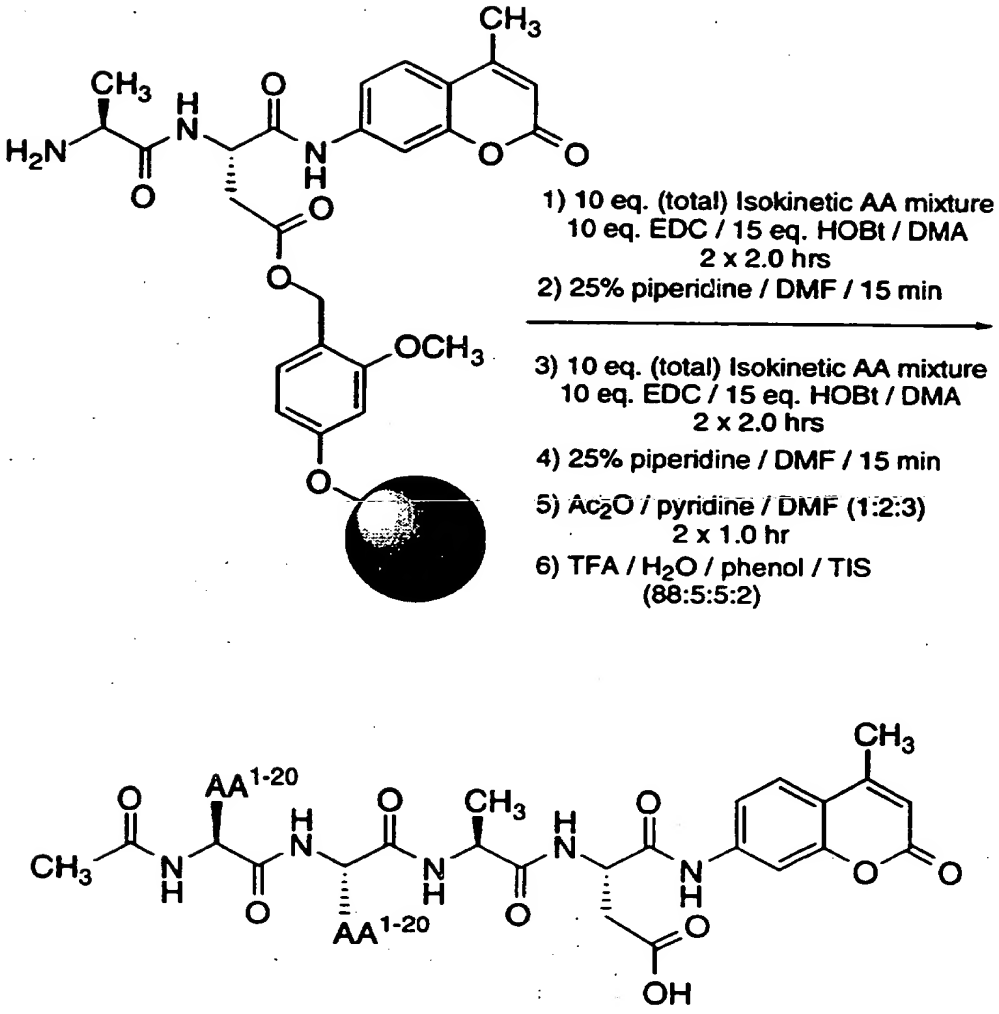


As depicted in Scheme 1, a P3 spatially addressed library may be prepared essentially as follows. N-allyloxycarbonyl-L-aspartic acid-α-aminomethylcoumarin is loaded onto a Rapp Polymere TentaGel S NH_2 resin containing the 4-(4-hydroxymethyl-3-methoxy-phenoxy)-butyric acid (HMPB) handle *via* the Mitsunobu reaction employing diisopropyl axodicarboxylate and triphenylphosphine (DIAD/TPP). After removal of the Alloc group using Pd(Ph3)4 and 1,3-dimethylbarbituric acid in dichloromethane, the resin is dried in a stream of nitrogen overnight. The following day, the resin is pre-swelled in DMA and drained. The isokinetic mixture of protected amino acids is prepared by dissolving the requisite amounts of each monomer in DMA along with HOBT. After complete dissolution occurred, EDC is added. The isokinetic mixture is then added to the resin followed by agitation for 2 hours. After draining the solution, the resin is washed with DMA and the entire procedure was repeated (double coupling). The resin is washed and the Fmoc was removed with piperidine/DMF. After further washing, the resin is transferred into 20 individual reaction vessels by the isopycnic slurry method (R. N. Zuckermann, et al., Int. J. Pept. Protein Res., 40, 479 (1992)). Position P3 is then "spatially addressed" by pre-activating the 20 individual amino acids with EDC/HOBT as described above followed by addition to the 20 reaction vessels (double coupling). After Fmoc removal, P4 substituents are installed by adding the isokinetic mixture of amino acids to each vessel. Following Fmoc removal, the N-terminus is acetylated with Ac2O/pyridine/DMF (double coupling). The resin bound mixtures are then twice cleaved for 30 minutes using TFA/H_2O/PhOH/TIS (88:5:5:2) . The cleavage solution is aged for 1 hour and 20 minutes before the solvent is removed *in vacuo*.

The tetrapeptide-AMC derivates are twice precipitated from cold Et_2O before being lyophilized from CH_3CN/H_2O (2:1).

SCHEME 2





As depicted in Scheme 2, the P_2 position may be spatially addressed in a manner essentially as described in Scheme 1 for spatially addressing the P_3 position.

The following examples are provided for the purpose of further illustration only and are not intended to be limitations on the disclosed invention.

EXAMPLE 1

Synthesis and Characterization of Positional Scanning Library

As a representative example, the P3 spatially addressed library was prepared as follows: Rapp Polymere TentaGel S NH_2 resin (2.5 g; 0.725mmol) containing the 4-(4-hydroxymethyl-3-methoxy-phenoxy)-butyric acid (HMPB) handle was pre-swelled in 31 mL of dry THF. The solvent was drained, and to the resin was added a solution of N-allyloxycarbonyl-L-aspartic acid-α-aminomethylcoumarin (2.71 g; 7.25 mmol; 10 equiv.) in THF (31 mL) and DMA (1 mL), followed by PPh_3 (952 mg; 3.63 mmol; 5 equiv.). Upon dissolution, neat diisopropyl azodicarboxylate (571 µL; 3.63 mmol; 5 equiv.) was added and the cartridge was rotated for 1 hour. After draining the solution, the resin was washed 3 times each with DMA, THF, IPA, and DCM. A 0.25 M 1,3-dimethylbarbituric acid solution in DCM (21 mL) was added to the resin (2.1 g; 0.61 mmol), followed by $Pd(PPh_3)_4$ (1.05 g; 0.9 mmol; 1.5 equiv.). After 2 hours of agitation, the resin was washed alternately with pyridine and AcOH (to remove excess palladium), then 3 times each with THF, THF/TEA (3:1), THF, IPA, and DCM. The resin was dried in a stream of nitrogen overnight. The following day, the resin (2.0 g; 0.58 mmol) was pre-swelled in DMA (25 mL) and drained. The isokinetic mixture of protected amino acids was prepared by dissolving the requisite amounts of each monmer (see "Isokinetic Mixture Information" Table) in DMA (25 mL) along with HOBT (2.35 g; 17.4 mmol; 15 equiv.) After complete dissolution occurred, EDC (2.22 g; 11.5 mmol; 10 equiv.) was added. The isokinectic mixture was then added to the resin followed by agitation for 2 hours. After draining the solution, the resin was washed with DMA and the entire procedure was repeated (double coupling). Exposure to 25% piperidine in DMF (25 mL) for 15 minutes was followed by washing with DMF, THF, iPrOH and DCM. The resin was then transferred into 20 individual reaction vessels by the isopycnic slurry method and washed with DMA.

Position P3 was then "spatially addressed" as follows: EDC (55 mg; 0.29 mmol; 10 equiv.) was weighed into 20 vials. The 20 amino acids (see "Spatially Addressed Position" Table) were weighed into

another 20 vials. To each amino acid vial was added DMA (1.5 mL). HOBT (2.35g; 17.3 mmol) was dissolved in DMA (20 mL) and one mL of this solution was added to each amino acid vial. One half of the amino acid/HOBT solution (1.25 mL) was then added to each EDC vial and in turn, added to the 20 sequentially numbered reactors containing the resin. Thus, each reactor contained 10 equivalents of EDC, 10 equivalents of one amino acid, and 15 equivalents of HOBT. After 2 hours of agitation, the resin was drained, washed with DMA and the procedure repeated, this time adding the EDC (55 mg; 0.29 mmol; 10 equiv.) to the balance of the amino acid/HOBT solution (1.25 mL). The resin was then washed with DMA and DCM. Exposure of each vessel to 25% piperidine in DMF for 15 minutes was followed by washing with DMF, THF, iPrOH and DCM. The isokinetic mixture was prepared as described previously and added to each reactor. Double coupling (2x2 hrs), Fmoc removal and washing as above was followed by capping the N-terminus with a (1:2:3) solution of Ac_2O/pyridine/DMF (2 x 1 hr). After washing with DMA, H_2O, THF, iPrOH, and DCM, cleavage and concomitant removal of the sidechain protecting groups was achieved by exposure to a degassed solution of 88:5:5:2 TFA/H_2O/PhOH/TIS (1 mL) for 30 minutes. The cleavage was repeated one time. The cleavage solution was aged for 1 hour and 20 minutes before the volatiles were removed *in vacuo*. The tetrapeptide-AMC derivates were twice precipititated from cold Et_2O before being lyophilized from CH_3CN/H_2O (2:1). The yields for the individual wells ranged from 30 to 49%. Each of the 60 samples were prepared as approximately 10 mM stocks in dimethyl sulfoxide (DMSO).

Is kinetics Mixture Information

AA	Molar %	MW	mmol needed	mg needed
Ala	3.32	311.33	0.193	60.0
D-Ala	3.32	311.33	0.193	60.0
Asp	3.43	411.45	0.199	81.9
Glu	3.56	425.48	0.207	87.9
Phe	2.47	387.43	0.143	55.5
Gly	2.82	297.31	0.164	48.6
His	3.49	619.72	0.202	125.4
Ile	16.98	353.42	0.985	348.0
Lys	6.09	468.55	0.353	165.4
Leu	4.85	353.42	0.281	99.5
Nle	2.25	353.42	0.130	46.0
Asn	5.23	596.68	0.304	181.1
Pro	4.23	337.37	0.245	82.8
Gln	5.21	610.7	0.302	184.4
Arg	6.37	664.98	0.370	245.8
Ser	2.72	383.44	0.158	60.5
Thr	4.68	397.47	0.271	107.8
Val	11.04	339.39	0.640	217.3
Tyr	4.05	459.54	0.235	107.8
Trp	3.90	526.59	0.226	119.0
Total	100.00		5.800	

Spatially Addr ss d Positi n

Tube	AA	MW	mmol needed	mg needed	mg-2 couplings
1	D-Ala	311.33	0.290	90.3	180.6
2	Ala	311.33	0.290	90.3	180.6
3	Arg	662.50	0.290	192.1	384.3
4	Asn	596.68	0.290	173.0	346.1
5	Asp	411.45	0.290	119.3	238.6
6	Gln	610.70	0.290	177.1	354.2
7	Glu	425.48	0.290	123.4	246.8
8	Gly	297.31	0.290	86.2	172.4
9	His	619.72	0.290	179.7	359.4
10	Ile	353.42	0.290	102.5	205.0
11	Leu	353.42	0.290	102.5	205.0
12	Lys	468.55	0.290	135.9	271.8
13	Nle	353.42	0.290	102.5	205.0
14	Phe	387.43	0.290	112.4	224.7
15	Pro	337.37	0.290	97.8	195.7
16	Ser	383.44	0.290	111.2	222.4
17	Thr	397.47	0.290	115.3	230.5
18	Trp	526.59	0.290	152.7	305.4
19	Tyr	459.54	0.290	133.3	266.5
20	Val	339.39	0.290	98.4	196.8

EXAMPLE 2

Preparation of Individual, Fluorogenic Substrate

A representative procedure for single pure substrate synthesis is as follows: Rapp Polymere TentaGel S NH_2 resin (1.0 g; 0.17 mmol) containing the 4-(4-hydroxymethyl-3-methoxy-phenoxy)-butyric acid (HMPB) handle was preswelled in 10 mL of dry THF. The solvent was drained, and to the resin was added a solution of N-allyloxycarbonyl-L-aspartic acid-α-aminomethylcoumarin (636 mg; 1.7 mmol; 10 eq.) in THF (10 mL) and DMA (300 µL), followed by triphenylphosphine (223 mg; 0.85 mmol; 5 eq.). Upon dissolution, neat diisopropyl azodicarboxylate (167 µL; 0.85 mmol; 5 eq.) was added and the cartridge was rotated for 1 hour. After draining the solution, the resin was washed 3 times each with DMA, THF, IPA, and DCM. A 0.25 M 1,3-dimethylbarbituric acid solution in DCM (10 mL) was added to the

resin, followed by $Pd(PPh_3)_4$ (500 mg; 0.43 mmol; 2.5 eq.). After 2 hours of agitation, the resin was washed alternately with pyridine and AcOH (to remove excess palladium), then 3 times each with THF, THF/TEA (3:1), THF, IPA, and DCM. To a solution of HOBT (345 mg; 2.55 mmol; 15 eq.) and N-α-Fmoc-N-im-trityl-L-histidine (1.05 g; 1.7 mmol; 10 eq.) in NMP (10 mL) was added HBTU (645 mg; 1.7 mmol; 10 eq.). This mixture was added to the resin followed by DIPEA (592 μL; 3.4 mmol; 20 eq.). After 1 hour, the resin was washed 3 times each with DMF, THF, IPA, and DCM. Exposure to 25% piperidine in DMF (10 mL) for 15 minutes followed by washing with DMF (5x) provided the free amine. Coupling and Fmoc removal as above employing the requisite amino acids, in this case N-α-Fmoc-L-glutamic acid γ-t-butyl ester and N-α-Fmoc-N-in-t-Boc-L-tryptophan respectively, provided the resin bound tetrapeptide. Final Fmoc removal was followed by capping the N-terminus with Ac_2O/pyridine/DMF (1:2:3) (10 mL; 2 x 1 hr). After washing with DMF, THF, IPA, and DCM, cleavage and concomitant removal of the sidechain protecting groups was achieved by exposure to a degassed solution of 88:5:5:2 TFA/H_2O/PhOH/TIS (10 mL) for 20 minutes. The cleavage was repeated and the volatiles removed *in vacuo*. After precipitation from cold Et_2O followed by lyophilization from CH_3CN/H_2O (2:1), purification *via* MPLC (Lobar LiChroprep RP-18 column; 5 min linear gradient from 1 to 5 mL/min [20% CH_3CN/H_2O containing 0.1% TFA], followed by a 20 min linear gradient to 25% CH_3CN/H_2O) provided after lyophilization 56 mg (37% yield) Ac-WEHD-AMC as a white solid. ^1H NMR (500 MHz, CD_3OD) δ 1.80-1.87 (m, 1H), 1.93-2.00 (m, 1H), 1.95 (s, 3H), 2.13-2.16 (complex m, 2H), 2.39 (d, J = 1.1 Hz, 3H), 2.95 (ABX, J_{AB}=16.9 Hz, J_{AX}=8.1 Hz, J_{BX}=5.5 Hz, $\Delta\nu_{AB}$=68.2 Hz, 2H), 3.10-3.30 (complex m, 4H), 4.13 (dd, J=8.8, 5.1 Hz, 1H), 4.58 (dd, J=7.7, 5.6 Hz, 1H), 4.62 (dd, J=7.7, 5.7 Hz, 1H), 4.80 (dd, J=8.0, 5.4 Hz, 1H), 6.19 (d, J=1.1 Hz, 1H), 6.95 (apparent td, J=7.5, 0.9 Hz, 1H), 7.05 (apparent td, J=7.6, 1.1 Hz, 1H), 7.15 (s, 1H), 7.29 (d, J=8.3 Hz, 1H), 7.33 (d, J=1.2 Hz, 1H), 7.48-7.51 (complex m, 2H), 7.62 (d, J=8.7 Hz, 1H), 7.80 (d, J=2.1 Hz, 1H), 8.71 (d, J=1.4 Hz, 1H); HRMS (FAB-POS) found *m/e* 785.2947 (MH^+ calcd for $C_{38}H_{41}N_8O_{11}$, 785.2895).

EXAMPLE 3

Synthesis and Purification of Ac-WEHD-CHO

Rapp Polymere TentaGel S NH_2 resin (2.0 g; 0.34 mmol) containing the 4-(4-hydroxymethyl-3-methoxy-phenoxy)-butyric acid (HMPB) handle was pre-swelled in 24 mL of dry THF. The solvent was drained, and to the resin was added a solution of N-α-Fmoc-N-im-trityl-L-histidine (2.1 g; 3.4 mmol; 10 equiv.) in THF (24 mL) and DMA (a few drops), followed by triphenylphosphine (444 mg; 1.7 mmol; 5 equiv.). Upon dissolution, neat diisopropyl azodicarboxylate (334 μL; 1.7 mmol; 5 equiv.) was added and the cartridge was rotated for 1.5 hour. After draining the solution, the resin was washed 3 times each with DMA, H_2O, THF, IPA, and DCM. Elimination of the Fmoc by exposure of the resin to 25% piperidine in DMF for 15 minutes was followed by washing the resin with DMF (5x). To a solution of HOBT (344 mg; 2.55 mmol; 7.5 equiv.) and N-α-Fmoc-L-glutamic acid γ-benzyl ester (780 mg; 1.7 mmol; 5 equiv.) in NMP (24 mL) was added HBTU (644 mg; 1.7 mmol; 5 equiv.). This mixture was added to the resin followed by DIPEA (592 μL; 3.4 mmol; 10 equiv.). After 1 hour, the resin was washed 3 times each with DMF, THF, IPA, and DCM. Fmoc removal and coupling as above employing N-α-Fmoc-N-in-t-Boc-L-tryptophan provided the resin bound tripeptide. Final Fmoc removal was followed by capping the N-terminus with a 1:2:3 solution of Ac_2O/pyridine/DMF (20 mL; 2 x 1 hr). After washing with DMF, THF, IPA, and DCM, the tripeptide was cleaved from the resin by exposure to a degassed solution of 88:5:5:2 TFA/water/phenol/ triisoproplysilane (20 mL) for 20 minutes. The cleavage was repeated one time. Removal of the cleavage cocktail *in vacuo* was followed by precipitation from cold diethyl ether. The amorphous solid was lyophilized from acetonitrile/water (2:1) providing Ac-WE(OBzl)H-OH•TFA (108 mg) in 44% yield. To a stirred solution of N-allyloxycarbonyl-4-amino-5-benzyloxy-2-oxotetrahydrofuran (22 mg; 0.078 mmol) and Ac-WE(OBzl)H-OH•TFA (67 mg; 0.093 mmol; 1.2 equiv.) in anhydrous THF (300 μL) and DMF (300 μL) under N_2 was added $(PPh_3)_2PdCl_2$ (2 mg). After aproximately 3-5 minutes, tri-n-

butyltin hydride (23 µL; 0.08 mmol; 1.1 equiv.) was added. Gas evolution occurred. An additional 1.1 equivalents of tri-n-butyltin hydride was added. After 15-20 minutes the reaction vessel was cooled to 0 °C followed by the addition of HOBT (21 mg; 0.156 mmol; 2 equiv.). After dissolution occurred, EDC (18 mg; 0.094 mmol; 1.2 equiv.) was added. After allowing the reaction mixture to reach ambient temperature over 2 hours, the crude reaction mixture was further diluted with DMF and purified *via* MPLC (Sephadex LH-20; DMF as the eluent). The DMF was removed *in vacuo* and the desired product was precipitated from diethyl ether/hexane to provide N-(N-acetyltyrosinyl-glutamyl(OBzl)-histidinyl)-4-amino-5-benzyloxy-2-oxotetrahydrofuran (36 mg) in 50 % yield. This penultimate intermediate (18.5 mg; 0.020 mmol) was dissolved in methanol (5 mL) and exposed to 20% $Pd(OH)_2$ (13 mg) under hydrogen for one hour at which time analytical HPLC (4x250mm LiChrospher 100 RP-18 5 µm particle size, 50/50 MeCN/H_2O containing 0.1% TFA, 1.0 mL/min) indicated complete consumption of the starting material (RT=8.6 minutes). Purification *via* MPLC (Lobar LiChroprep RP-18; 15% acetonitrile/water containing 0.1% trifluoroacetic acid) provided after lyophilization Ac-WEHD-CHO (6.6 mg) in 45% yield. ^1H NMR (500 MHz, CD_3OD) δ 1.73-1.76 (m, 1H), 1.86-1.91 (m, 1H), 1.88 (s, 3H), 2.07-2.10 (complex m, 2H), 2.44-2.50 (m, 1H), 2.58-2.63 (m, 1H), 2.96-3.01 (1/2ABX, J_{AB}=15.3 Hz, J_{AX}=7.5 Hz, 1H), 3.06-3.10 (1/2ABX, J_{AB}=14.7 Hz, J_{AX}=7.7 Hz, 1H), 3.13-3.28 (complex m, 2H), 4.08 (ddd, J=7.3, 5.2, 2.0 Hz, 1H), 4.19-4.25 (m, 1H), 4.50-4.60 (complex m, 3H), 6.93 (apparent td, J=7.4, 1.0 Hz, 1H), 7.00 (apparent td, J=7.5, 1.0 Hz, 1H), 7.09 (s, 1H), 7.24 (complex m, 2H), 7.49 (d, J=7.7 Hz, 1H), 8.62 (dd, J=3.9, 1.4 Hz, 1H); HRMS (FAB-POS) found *m/e* 612.2400 (MH^+ calcd for $C_{28}H_{34}N_7O_9$, 612.2418).

EXAMPLE 4

Preparation of Active ICE

The method used for production of recombinant protease involves folding of active enzyme from its constituent large and small

subunits which are expressed separately in E. coli. The 20 kDa (p20) and 10 kDa (p10) subunits of ICE were individually expressed in E. coli BL21(DE3)pLysS cells and recombinant protein expression was induced by overnight growth in the presence of 1 mM IPTG. Cells were harvested, washed and broken in the presence of protease inhibitors then inclusion bodies were isolated and solubilized in 6 M guanidine-hydrochloride. To obtain active enzyme, the individual subunits were rapidly diluted to a final concentration of 100 mg/ml at ambient temperature into a solution containing 100 mM Hepes, 10% sucrose, 10 mM DTT, 1% TX-100, pH 7.5 and Sepharose-Ac-YVAD-CHO (0.05 ml resin/ml solution) [20, 28]. After stirring the solution for three days the resin was harvested and washed exhaustively with 100 mM Hepes, 10% sucrose, 0.1% CHAPS, pH 7.5. The enzyme was eluted by treatment with 100 μM Ac-YVAD-CHO for 24 hours at ambient temperature. Inhibitor was removed by treatment with hydroxylamine and oxidized glutathione as described by Thornberry, N. A., Methods in Enzymology, 244, 615-631 (1994).

EXAMPLE 5

Measurement of Kinetic Constants

Measurement of k_{cat}/K_m for individual tetrapeptide-AMC substrates: Liberation of AMC from each substrate was monitored continuously in a Gilford Fluoro IV Spectrofluorometer using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Reactions were initiated by adding 10-60 nM enzyme to an assay mixture containing 0.5 μM substrate in 100 mM Hepes, 10% sucrose, 0.1% CHAPS, 0.1% ovalbumin, 10 mM DTT, pH 7.5, and 25°C. Data were fit by nonlinear regression to a first-order rate equation ($AMC = A*exp^{-kobs*t} + B$) to obtain the rate constant for hydrolysis of substrate ($k_{obs} = k_{cat}*E_t/K_m$). This rate constant was corrected for enzyme concentration to produce values for k_{cat}/K_m. This data is presented in Table 1.

Table 1

Comparison of Substrates for ICE

Substrate	$10^{-5} \times k_{cat}/K_m$ ($M^{-1}s^{-1}$)
Ac-WEHD-AMC	33.4 ± 0.3
Ac-WVHD-AMC	15.7 ± 0.1
Ac-YEHD-AMC	9.56 ± 0.20
Ac-WEAD-AMC	7.55 ± 0.07
Ac-YVHD-AMC	2.81 ± 0.14
Ac-WVAD-AMC	2.41 ± 0.60
Ac-YEAD-AMC	1.85 ± 0.07
Ac-YVAD-AMC	0.66 ± 0.14
pro-IL-1β	1.5

EXAMPLE 6

Measurement of K_i for Ac-WEHD-CHO

The continuous, fluorometric assay employed for these studies has been previously described (Thornberry, N. A., et al., Nature, 356, 768-774 (1992); Thornberry, N. A., Methods in Enzymology, 244, 615-631 (1994)). All reactions were performed under standard reaction conditions (100 mM Hepes, 10% sucrose, 0.1% CHAPS, 0.1% ovalbumin, 10 mM DTT, pH 7.5, and 25°C) using homogeneous enzyme. Reactions were monitored continuously in a Gilford Fluoro-IV fluorometer using an excitation wavelength of 380 nM and an emission wavelength of 460 nm. To measure the association rate constant (k_{on}), enzyme was added to reaction mixtures containing 1 x K_m Ac-YVAD-AMC and various concentrations of inhibitor. The dissociation rate constant (k_{off}) was determined by preincubation of enzyme and inhibitor (25 nM ICE, 125 nM Ac-WEHD-CHO), followed by 500-fold dilution into a reaction mixture containing 100xK_m levels of substrate. The overall dissociation constant, K_i, was calculated from the observed rates of association and dissociation according to the equations developed by Morrison for analysis of slow and tight-binding inhibitors (Morrison, et al., Adv.

Enzymol. Relat. Areas Mol. Biol., 61, 201-301 (1988)). This data is presented in Table 2.

Table 2

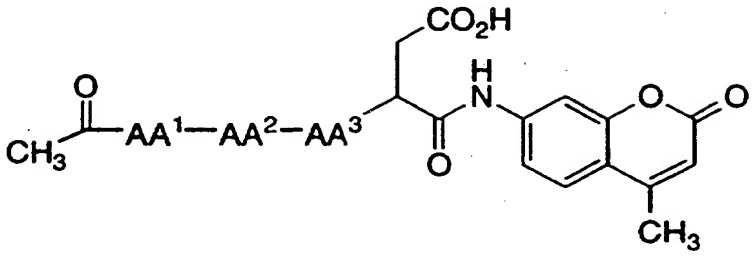
Inhibition of ICE by Peptide Aldehydes

	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_i (pM)
Ac-YVAD-CHO	3.8×10^5	2.9×10^{-4}	760
Ac-WEHD-CHO	2.6×10^5	1.5×10^{-5}	56
	Selectivity		14-fold

While the invention has been described and illustrated with reference to certain particular embodiments thereof, those skilled in the art will appreciate that various adaptations, changes, modifications, substitutions, deletions, or additions of procedures and protocols may be made without departing from the spirit and scope of the invention. For example, effective dosages other than the particular dosages as set forth herein above may be applicable as a consequence of variations in the responsiveness of the mammal being treated for any of the indications with the compounds of the invention indicated above. Likewise, the specific pharmacological responses observed may vary according to and depending upon the particular active compounds selected or whether there are present pharmaceutical carriers, as well as the type of formulation and mode of administration employed, and such expected variations or differences in the results are contemplated in accordance with the objects and practices of the present invention. It is intended, therefore, that the invention be defined by the scope of the claims which follow and that such claims be interpreted as broadly as is reasonable.

WHAT IS CLAIMED IS:

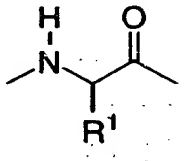
1. A compound of the formula I:



I

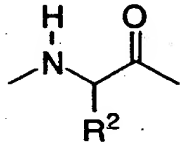
wherein:

AA^1 is an amino acid of formula AI:



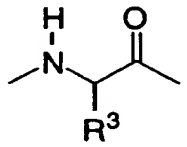
AI

AA^2 is an amino acid of formula AII:



AII

AA^3 is an amino acid of formula AIII:



AIII

wherein R^1, R^2 and R^3 are each independently selected from the group consisting of:

(a) hydrogen,

(b) C_{1-6}alkyl or substituted C_{1-6}alkyl, wherein the substituent is selected from:

(1) hydroxy,

(2) halo,

(3) -S-C_{1-4}alkyl,

(4) -SH

(5) C_{1-6}alkylcarbonyl,

(6) -CO_2H,

(7) -$CONH_2$,

(8) amino carbonyl amino,

(9) C_{1-4}alkylamino, wherein the alkyl is optionally substituted with hydroxy, and the amino is substituted with hydrogen or carbobenzyloxy,

(10) guanidino,

(11) C_{1-4}alkyloxy,

(12) phenyl C_{1-4}alkyloxy,

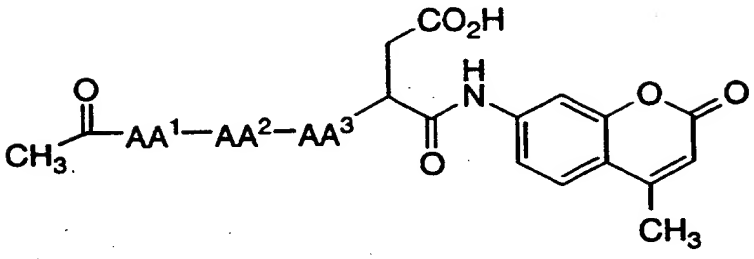
(13) phenyl C_{1-4}alkylthio,

(14) C_{1-6}alkyloxycarbonyl, and

(c) aryl-C_{1-6} alkyl, wherein aryl is phenyl, 1- or 2-naphthyl, 9-anthracyl, or 2-, 3- or 4- pyridyl, and wherein the aryl may be mono and di-substituted, wherein the substituent is independently selected from: C_{1-6}alkyl, halo, hydroxy, C_{1-6}alkyl amino, C_{1-6}alkoxy, C_{1-6}alkylthio, and C_{1-6}alkylcarbonyl;

and pharmaceutically acceptable salts thereof.

2. A compound of the formula:



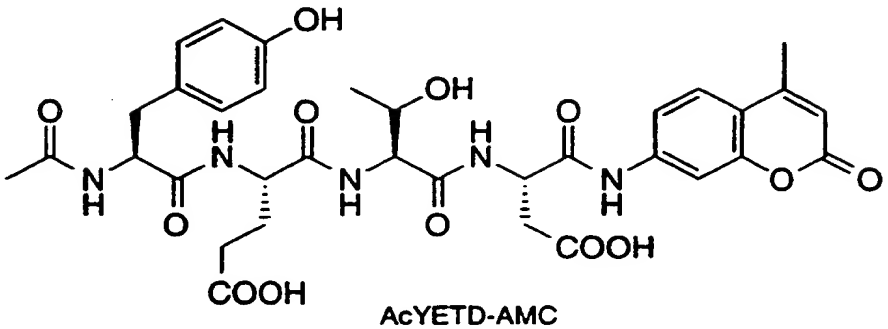
I

wherein:

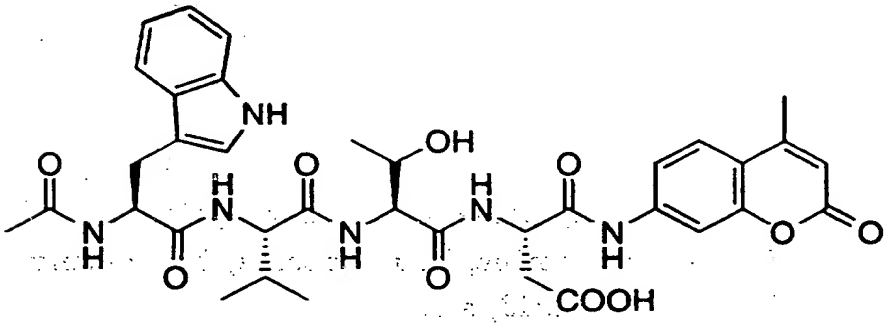
AA^1, AA^2 and AA^3 are each independently selected from the group consisting of: the L- and D- forms of the amino acids including glycine, alanine, valine, leucine, isoleucine, norleucine, serine, threonine, aspartic acid, asparagine, glutamic acid, glutamine, lysine, hydroxy lysine, histidine, proline, hydroxy proline, arginine, phenylalanine, tyrosine, tryptophan, cysteine, methionine, β-alanine, ornithine, homoserine, homotyrosine, homophenylalanine, aminoisobutyric acid, and citrulline,

and pharmaceutically acceptable salts thereof.

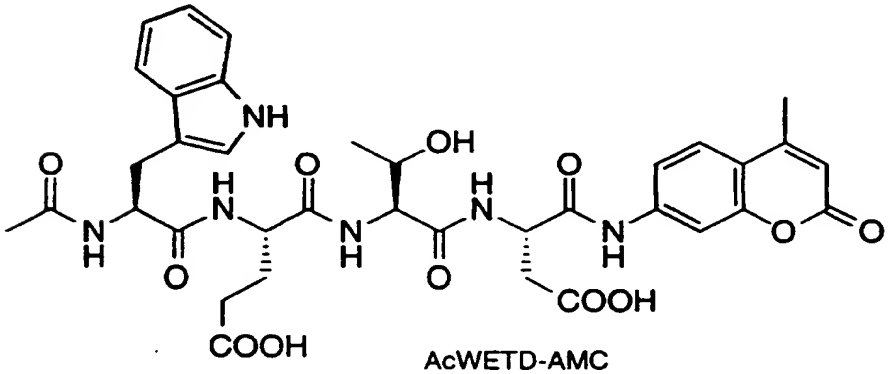
3. A compound which is selected from the group consisting of:



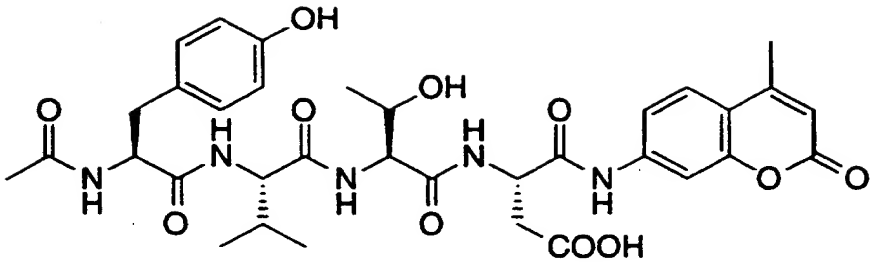
AcYETD-AMC



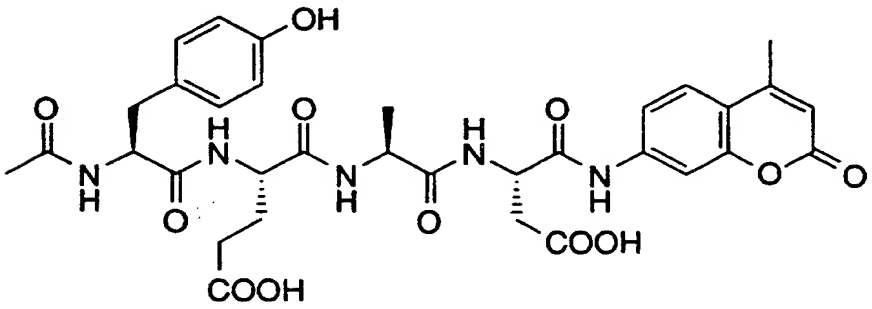
AcWVTD-AMC



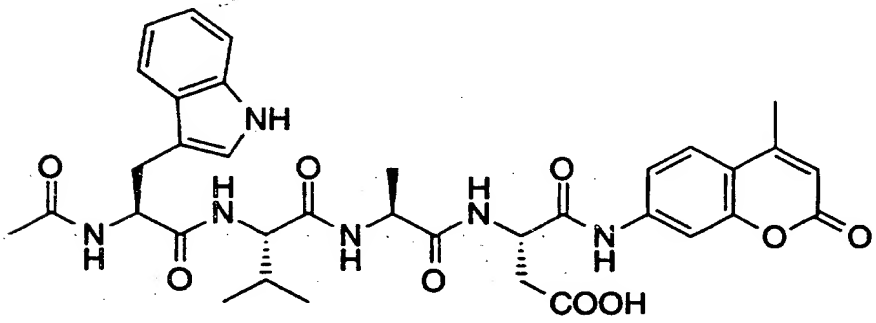
AcWETD-AMC



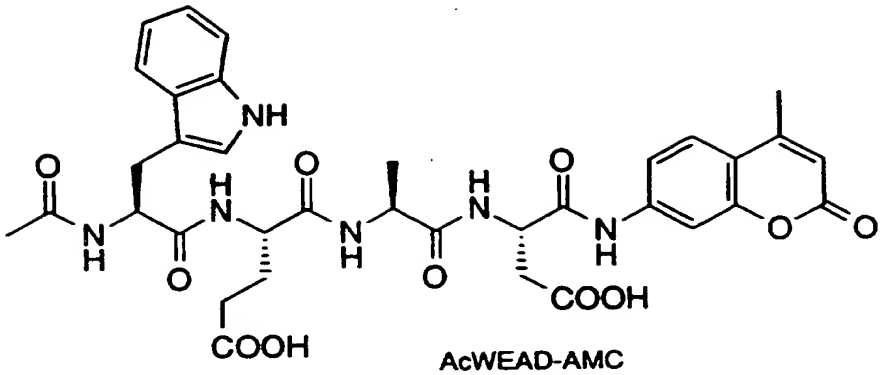
AcYVTD-AMC



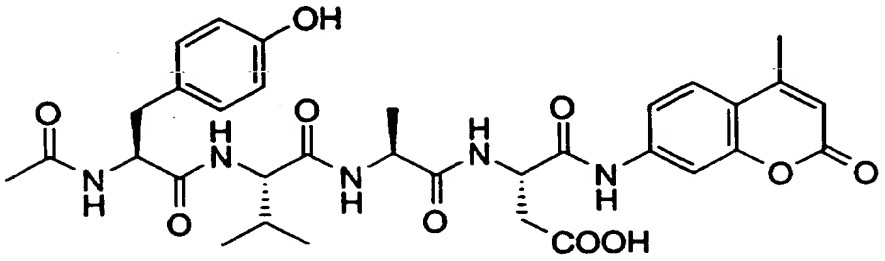
AcYEAD-AMC



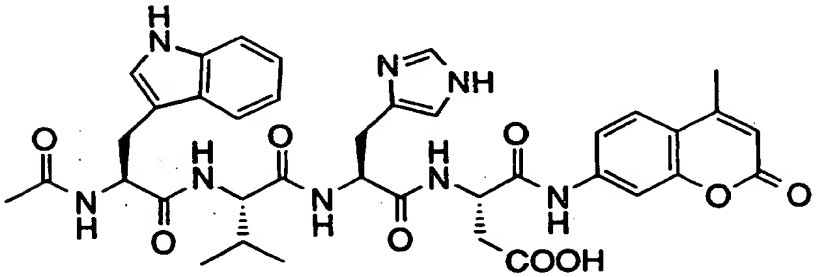
AcWVAD-AMC



AcWEAD-AMC



AcYVAD-AMC



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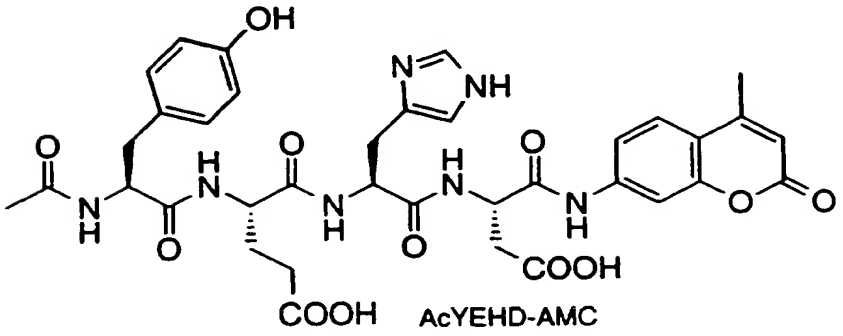

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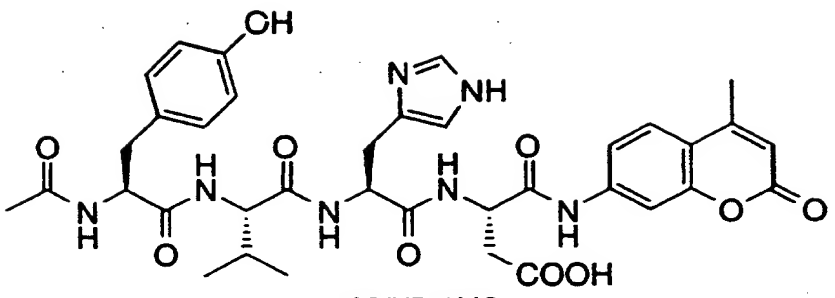
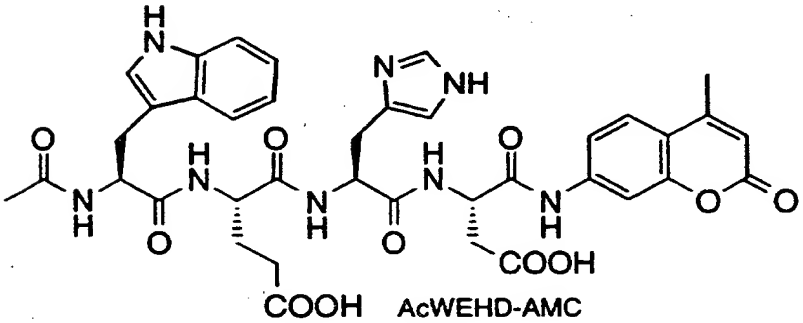
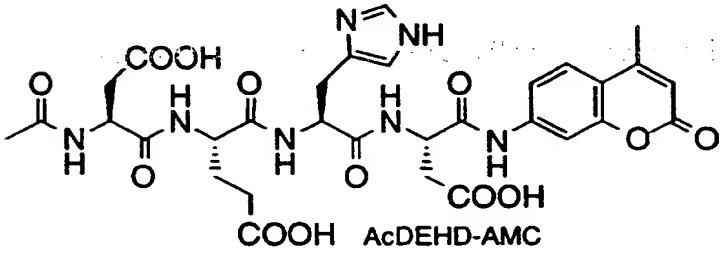


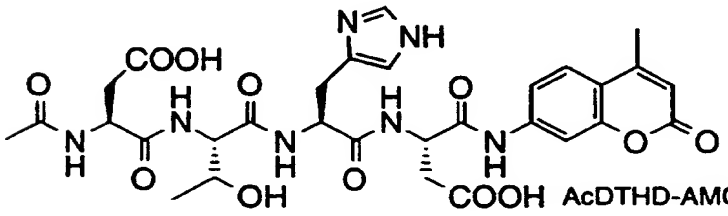
AcYVHD-AMC



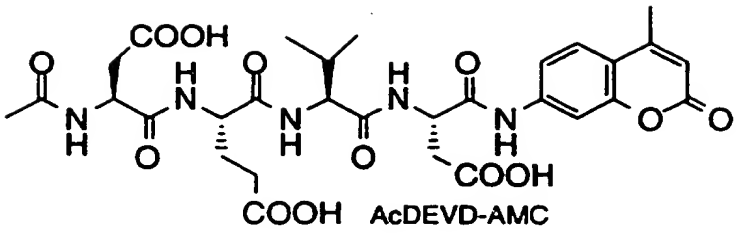
AcWEHD-AMC



AcDEHD-AMC

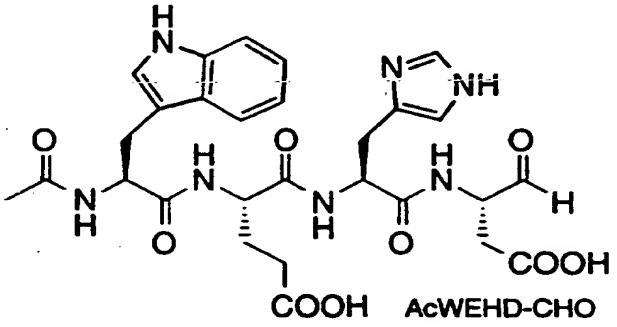


AcDTHD-AMC



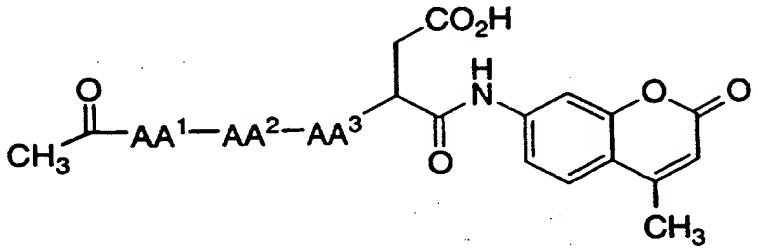
and pharmaceutically acceptable salts thereof.

4. A compound which is:



and pharmaceutically acceptable salts thereof.

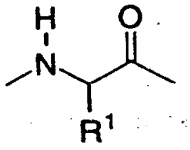
5. A combinatorial library comprising a mixture of at least 50 compounds of the formula I:



I

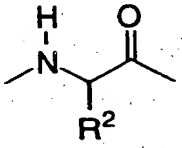
wherein:

AA^1 is an amino acid of formula AI:



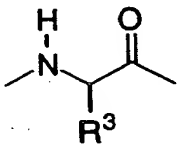
AI

AA^2 is an amino acid of formula AII:



AII

AA^3 is an amino acid of formula AIII:



AIII

wherein R^1, R^2 and R^3 are each independently selected from the group consisting of:

(a) hydrogen,

(b) C_{1-6}alkyl or substituted C_{1-6}alkyl, wherein the substituent is selected from:

(1) hydroxy,

(2) halo,

(3) -S-C_{1-4}alkyl,

(4) -SH

(5) C_{1-6}alkylcarbonyl,

(6) -CO_2H,

(7) -$CONH_2$,

(8) amino carbonyl amino,

(9) C_{1-4}alkylamino, wherein the alkyl is optionally substituted with hydroxy, and the amino is substituted with hydrogen or carbobenzyloxy,

(10) guanidino,

(11) C_{1-4}alkyloxy,

(12) phenyl C_{1-4}alkyloxy,

(13) phenyl C_{1-4}alkylthio,

(14) C_{1-6}alkyloxycarbonyl, and

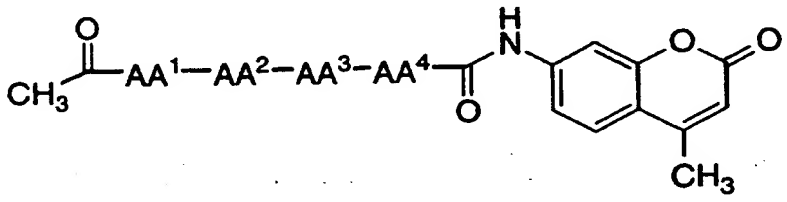
(c) aryl-C_{1-6} alkyl, wherein aryl is phenyl, 1- or 2-naphthyl, 9-anthracyl, or 2-, 3- or 4- pyridyl, and wherein the aryl may be mono and di-substituted, wherein the substituent is independently selected from: C_{1-6}alkyl, halo, hydroxy, C_{1-6}alkyl amino, C_{1-6}alkoxy, C_{1-6}alkylthio, and C_{1-6}alkylcarbonyl;

and pharmaceutically acceptable salts thereof.

6. The combinatorial library of Claim 5 wherein the mixture comprises at least 100 compounds of the formula I.

7. The combinatorial library of Claim 6 wherein the mixture comprises at least 200 compounds of the formula I.

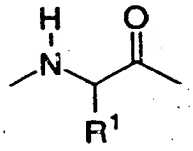
8. A method for using a combinatorial library to identify the substrate specificity of a cysteine or a serine protease enzyme, wherein the combinatorial library is comprised of a mixture of compounds of the formula II:



II

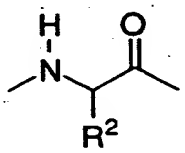
wherein:

AA^1 is an amino acid of formula AI:



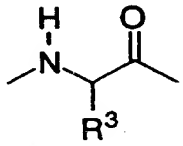
AI

AA^2 is an amino acid of formula AII:



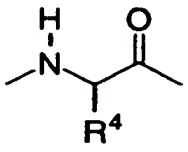
AII

AA^3 is an amino acid of formula AIII:



AIII

AA^4 is an amino acid of formula AIV:



AIV

wherein R^1, R^2, R^3 and R^4 are each independently selected from the group consisting of:

(a) hydrogen,

(b) C_{1-6}alkyl or substituted C_{1-6}alkyl, wherein the substituent is selected from:

(1) hydroxy,

(2) halo,

(3) -S-C_{1-4}alkyl,

(4) -SH

(5) C_{1-6}alkylcarbonyl,

(6) $-CO_2H$,

(7) $-CONH_2$,

(8) amino carbonyl amino,

(9) C_{1-4}alkylamino, wherein the alkyl is optionally substituted with hydroxy, and the amino is substituted with hydrogen or carbobenzyloxy,

(10) guanidino,

(11) C_{1-4}alkyloxy,

(12) phenyl C_{1-4}alkyloxy,

(13) phenyl C_{1-4}alkylthio,

(14) C_{1-6}alkyloxycarbonyl, and

(c) aryl-C_{1-6} alkyl, wherein aryl is phenyl, 1- or 2-naphthyl, 9-anthracyl, or 2-, 3- or 4- pyridyl, and wherein the aryl may be mono and di-substituted, wherein the substituent is independently selected from: C_{1-6}alkyl, halo, hydroxy, C_{1-}

6alkyl amino, C_{1-6}alkoxy, C_{1-6}alkylthio, and C_{1-6}alkylcarbonyl;

and pharmaceutically acceptable salts thereof.

9. The method of Claim 8 wherein the compounds of formula II AA^4 is aspartic acid.

10. A method for identifying the substrate specificity of a cysteine or a serine protease enzyme which comprises the steps of:

(a) providing a number of sets of soluble, unsupported mixed oligopeptides bearing a spectrophotometric or fluorogenic leaving group, wherein the number of sets is equal to the number of amino acid positions within each oligopeptide,

wherein each set of oligopeptides is comprised of subsets of oligopeptides, wherein each subset of oligopeptides is comprised of oligopeptides in which a predetermined position of the oligopeptide chain contains a fixed amino acid and in which the remaining positions of the oligopeptide chain contain a mixture of amino acids present in approximately equimolar concentrations;

(b) separately mixing each subset from one of the sets with a cysteine or a serine protease enzyme in an aqueous media, separately determining the production of the spectrophotometric or fluorogenic leaving group, and ascertaining the one or more subsets of the set that provide for the production of the spectrophotometric or fluorogenic leaving group; and

(c) repeating step (b) using the subsets from each of the remaining sets, wherein the identity and position of the amino acid residue of each one or more subsets of the set that provide for the production of the spectrophotometric or fluorogenic leaving group gives the substrate specificity of the cysteine or the serine protease enzyme.

The Patent Office

Application No: GB 9803559.5
Claims searched: 1-3 & 5-9

Examiner: Dr. J. Houlihan
Date f search: 21 August 1998

Patents Act 1977
Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.P):

Int Cl (Ed.6):

Other: CAS ONLINE

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
X	EP 0533226 A2 (MERCK & CO.) Example 8B	1-3
X	WO 96/33268 A1 (MERCK & CO.) Page 34 lines 5-15	1-3
X	WO 94/00154 A1 (MERCK & CO.) Page 48 Step D	1-3
P/X	Chem. & Biol. Vol. 4 1997. Rano T A *et. al.* "*A combinatorial approach for determining protease specificities:application to interleukin-1β converting enzyme (ICE)*". pages 149-155, see especially results page 150 lines 1-6 & Fig. 1	1-3 & 5-9
X	Chem. Pharm. Bull. Vol. 43 (8) 1995. Taguchi H *et. al.* "*Amino acids and peptides. XL. Synthesis of Ac-Tyr-Val-Ala-Asp-MCA using newly developed acetylating agent*". pages 1336-1339	1-3
A	J. Biol. Chem. Vol. 270 (39) 1995. Jean F *et. al.* "*Fluorescent peptidyl substrates as an aid in studying the substrate specificity of human prohormone convertase PC1 and human furin and designing a potent irreversible inhibitor*". pages 19225-31	

X Document indicating lack of novelty or inventive step
Y Document indicating lack of inventive step if combined with one or more other documents of same category.
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A Document indicating technological background and/or state of the art.
P Document published on or after the declared priority date but before the filing date of this invention.
E Patent document published on or after, but with priority date earlier than, the filing date of this application.